Seasonality of the antibiotic resistance gene blaCTX-M in temperate Lake Maggiore

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Seasonality of the beta lactamase gene *bla*$_{CTX-M}$ in temperate Lake Maggiore

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Abstract

The antibiotic resistance gene *bla*$_{CTX-M}$, responsible of the resistance to cephalosporins, has been detected in microbes from hospitals to open waters. We studied the seasonality and stability of *bla*$_{CTX-M}$ in Lake Maggiore over three years and the role of potential inputs of allochthonous bacteria and/or antibiotic pollution in promoting its occurrence. *bla*$_{CTX-M}$ was mainly present from January to July in the pelagic microbial community and the gene occurrence was significantly related to low water temperature. To evaluate its temporal stability in the bacterial community over a short period, we measured *bla*$_{CTX-M}$ daily over the course of six days. The gene was below the limit of quantification except for one sampling when its abundance peaked, suggesting a point contamination. The bacterial community of the lake in which *bla*$_{CTX-M}$ was detected suggests that at least two distinct bacterial populations contained the gene. The occurrence of known *bla*$_{CTX-M}$ containing genera and the occurrence of the gene, however, did not overlap. Furthermore, the experimental addition of cefotaxime to lake water incubations did not promote abundance of the gene. These data imply that

26 *bla*$_{CTX-M}$ was present in the environmental microbial community. Increases of gene abundances were
27 likely caused by environmental parameters other than antibiotic contamination.

28 **Keywords**
29 - Antibiotic resistance gene
30 - *bla*$_{CTX-M}$
31 - Expanded Spectrum Cephalosporins
32 - Environment
33 - Lake
34 - Bacterial community

35

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41

INTRODUCTION

Expanded Spectrum Cephalosporins (ESCs) were introduced in medical treatment in the 1980's because of their enhanced bactericidal effect on Gram-negative bacteria, which was uncommon in older cephalosporins. The subsequent massive clinical use of ESCs caused a selective pressure on treated bacteria resulting in the rise of extended-spectrum β-lactamase enzymes (ESBLs), conferring resistance to this antibiotic class (D'Andrea et al., 2013). *Enterobacteriaceae* positive for ESBLs are becoming more frequent in clinical and veterinary settings, constituting a major threat for healthcare and a growing cost for the national health systems (Hernández et al., 2012). ESBLs that are active against broad spectrum beta-lactams (i.e. cefotaxime and ceftazidime) (Tacão et al., 2012) are grouped in three main types; TEM, SHV and CTX-M (Liao et al., 2017). The corresponding resistance genes (*bla* genes) are commonly detected in clinical and environmental settings, among which *bla*$_{\text{CTX-M}}$ seems to be the most widespread (D'Andrea et al., 2013). This gene has been found in food products, pets and farm animals (summarized in (Canton and Coque, 2006)), in bacterial and phage DNA from wastewaters (Colomer-Lluch et al., 2014) and sludge (Calero-Cáceres et al., 2014), in freshwater microbial communities (Devarajan et al., 2015; Di Cesare et al., 2015) and in *Escherichia coli* isolated from research stations in Antarctica (Hernández et al., 2012). It can be hypothesized that the mobilization of *bla*$_{\text{CTX-M}}$ allows its constitutive presence in such different settings. In fact, the genetic context of *bla*$_{\text{CTX-M}}$ is commonly composed by mobile elements like insertion sequences (Canton and Coque, 2006), transposons (Guo et al., 2016), and conjugative plasmids (Liu et al., 2017). Furthermore, CTX-M beta-lactams are often found in hospital-related strains harboring other clinically relevant ARGs (i.e. *mcr*-1, (Quan et al., 2017)), which makes of *bla*$_{\text{CTX-M}}$ a common gene in multi-drug resistant bacteria.

In temperate areas, *bla*$_{\text{CTX-M}}$ is frequently found in freshwater lakes and rivers (Czekalski et al., 2015; Di Cesare et al., 2017; Di Cesare et al., 2015; Zurfluh et al., 2013). This gene seems to mainly enter freshwater systems through urban wastewater discharge (Czekalski et al., 2012; Di Cesare et al., 2016). The analysis of *bla*$_{\text{CTX-M}}$ harboring *E. coli* suggests that the strains found in

68 aquatic environments and in wastewaters are related to those in hospitals of the same geographical
69 area (Abgottspon et al., 2014; Geser et al., 2012; Varela et al., 2015). Thus, in order to fully
70 understand the cycle of *bla*$_{CTX-M}$, it is necessary to consider clinical settings as well as the
71 environment. In fact, *bla*$_{CTX-M}$ seems to be a particularly interesting model-gene to study the role of
72 the environment in the spread of ARGs (Eckert et al., 2018).

73 In a previous study, we found that *bla*$_{CTX-M}$ is common within the pelagic microbial
74 community of Lake Maggiore, but, in contrast to other tested ARGs (i.e. *sul*II and *tet*A), it was only
75 detected during some months over the course of 18 months (Di Cesare et al., 2015). Since *bla*$_{CTX-M}$
76 might either be associated with allochthonous or autochthonous bacteria, different scenarios might
77 explain the variations in the occurrence of the gene. In the first case, the gene might be continuously
78 seeded by allochthonous bacteria into the lake through wastewater treatment plant effluents.
79 Variations in the abundance of *bla*$_{CTX-M}$ in the lake would then arise from seasonal variations in the
80 use of antibiotics in human or veterinary medicine (i.e. higher consumption in winter (Van Boeckel
81 et al., 2014)). In the second case, the gene might be carried by members of the natural bacterial
82 community of the lake that follow specific seasonal patterns. Here, the abundance of *bla*$_{CTX-M}$ would
83 be influenced by the growth and mortality of the respective bacterial population(s), whereby selection
84 might either not act on the resistance gene at all (but on other properties of the bacteria) or favor the
85 bacteria carrying the gene through antibiotic contamination of water.

86 The aim of this study was to evaluate the seasonality and stability of *bla*$_{CTX-M}$ in Lake
87 Maggiore (Italy-Switzerland) and the role of potential contamination with bacteria and/or antibiotic
88 on its occurrence. The potential seasonality of *bla*$_{CTX-M}$ was evaluated using monthly data over the
89 span of three years. Sampling was carried out at two sites, a fully pelagic site in the center of the lake
90 and a coastal station in a lateral basin. We assumed that the coastal station (facing the largest city on
91 the lake) was more sensitive to punctual contaminations. In addition, a daily sampling campaign over
92 the course of a week was carried out to evaluate the short-term variability of the abundance of *bla*$_{CTX-M}$. We evaluated bacterial community data and searched for increased abundances of known *bla*$_{CTX-}$

94 M containing bacterial genera, more sensitive to point contamination. To test whether contamination
95 with antibiotic itself could promote the abundance of the gene we made an enrichment experiment
96 using cefotaxime added to the microbial community of Lake Maggiore, and measured gene
97 abundance after exposure to the antibiotic.
98
99 **MATERIALS AND METHODS**
100 Monitoring of Lake Maggiore
101 Lake Maggiore was sampled monthly from Jan 2013 – Dec 2015 at the pelagic station of Ghiffa
102 (45°56'57''N, 8°38'8''E) and the coastal station of Pallanza (45°55'28''N, 8°32'45''E) as described
103 elsewhere, and here we also use the data from January 2013 to June 2014 which are already published
104 therein (Di Cesare et al., 2015). In order to evaluate short-term changes in gene abundances samples
105 were taken daily at the coastal station from June 2nd to 7th 2014. A water sampler that collects a 5L
106 sample integrating water from 20m depth to the surface was used for all samples(Bertoni et al., 2010).
107 Additionally 5 mL of lake water were fixed with formaldehyde solution to a final concentration of
108 1.5% (vol/vol) for flow cytometry. The samples were kept at 4°C until analysis.
109 Water temperature was measured using a multiparametric probe at the pelagic station (OS 316,
110 Idronaut, Italy). Air temperature at the specific date were taken from the website *ilmeteo.it*, using
111 "Verbania" as a location. The samples were kept at 4°C in the dark until further processing for a
112 maximum of 24h.
113 Each sample was immediately pre-filtered through a 10 µm size net and then (300-600 mL) filtered
114 on 0.22 µm size polycarbonate filters. Filters were stored at -20°C until DNA extraction.
115
116 Enrichment experiment
117 To evaluate the impact of antibiotics on the occurrence of the gene enrichment experiments were
118 conducted by removing bacterial predators by filtration and dilution of the community with bacteria-
119 free lake water, in order to promote the growth of fast-growing bacteria (Neuenschwander et al., 2015;

120 Zeder et al., 2009). This was done because we assumed that many antibiotic-resistant bacteria entering
121 the lake might be fast-growing but sensitive to predation by heterotrophic flagellates and thus might
122 thrive better in such a setting (González et al., 1992; Wanjugi and Harwood, 2013).
123 In detail, on April 16th 2016, surface water samples were collected manually from the shore at the
124 coastal station. Nine times 700 mL of water were autoclaved in 1 L glass bottles (prior sterilized at
125 about 500°C). In parallel, 2 L of water were filtered through a 0.8 µm pore size polycarbonate filter
126 (47 mm diameter) to remove large bacterial predators (Neuenschwander et al., 2015; Zeder et al.,
127 2009). Thereafter, 70 mL of 0.8 µm pre-filtered lake water (inoculum) was mixed with 700 mL of
128 autoclaved lake water to create the enrichments.
129 In order to increase abundance of *bla*$_{CTX-M}$ harbouring bacteria we added 1µg mL^{-1} of cefotaxime to
130 triplicate incubations, the same used to select ESBL-bacteria (Santanirand et al., 2011) and 10^6 times
131 higher than that found in an aquatic environment previously (Han and Lee, 2017), thus mimicking a
132 high antibiotic release. Two different types of controls were conducted in triplicate: one enrichment
133 without any antibiotics and one with 1µg mL^{-1} of ampicillin, in order to provide an antibiotic similar
134 to cefotaxime but not specifically selecting for *bla*$_{CTX-M}$. All enrichments were incubated at 20°C in
135 the dark for 4 days.
136 Samples for DNA analysis were taken from the original inoculum and at day 4 of the incubations by
137 filtration of 500 mL as described above. An aliquot of 2 mL from each enrichment were sampled on
138 day 0, day 1 and day 4 of the experiment for flow cytometric analysis and fixed with formaldehyde
139 solution to a final concentration of 1.5% (vol/vol). Another aliquot of 2 mL from the enrichment with
140 cefotaxime were twenty or fifty fold diluted and 100µL of these diluted samples were plated on R2A
141 agar medium (it was chosen as growth medium since it was designed for aquatic bacteria)
142 supplemented with 10 µg mL^{-1} of cefotaxime, the plates were incubated at 20°C for 72 h. The samples
143 were kept at 4°C until analysis.
144
145 Flow Cytometry

146 Total bacterial counts were performed using an Accuri C6 (BD) flow-cytometer. For the analysis,
147 100 µL of each sample were stained with SYBR Green I (Thermo Scientifics) to a final concentration
148 1% and incubated for 15 min in the dark at room temperature before analysis. Data analysis was
149 performed with the software provided with the instrument. Events that showed significant
150 fluorescence (FL1) and size (SSC), and were thus distinguishable from the background, were
151 manually selected (gated) in the cytograms and automatically counted.
152
153 DNA extraction, $bla_{CTX\text{-}M}$ detection by PCR and 16S rDNA and $bla_{CTX\text{-}M}$ quantification by qPCR
154 The molecular analysis were been carried out on the isolates grew on the plates and from the filters,
155 both from the enrichment experiments. In detail, 94 colonies selected because of different color and
156 morphology, were processed for the DNA extraction by the so-called Freeze-Thaw method, thus the
157 DNA samples were quantified using a fluorimeter Qubit, with a dsDNA HS Assay Kit provide
158 according to the manufacturer's protocol and analysed for the presence of the $bla_{CTX\text{-}M}$ gene by PCR.
159 Reactions were carried out in a total volume of 25µl containing 2µL of DNA, 0.5µM of each primer
160 (5'-ATGTGCAGYACCAGTAARGTKATCGC-3' and 5'-
161 TGGGTRAARTARGTSACCAGAAYCAGCGG-3', Boyd et al.2004) using GoTaq Green Master
162 Mix (Promega) according to the manufacturer's protocol. The PCR program was 95°C for 3 min, 30
163 cycles of 95°C for 30 seconds, 58°C for 60 seconds, 72°C for 60 seconds and a final extension of
164 72°C for 7 min.
165 Two sections of each filter were processed separately for the DNA extraction using Ultra Clean
166 Microbial DNA Isolation Kits (MoBio Laboratories) following the manufacture's instruction.
167 The DNA samples were 1:2 diluted and analyzed for the abundance of 16S rDNA using the primer
168 Bact1369F andProk1492R (Suzuki et al., 2000) as modified in (Di Cesare et al., 2015) and $bla_{CTX\text{-}M}$
169 primers as published in (Marti et al., 2013) by qPCR following the protocols and programs already
170 described elsewhere by using a CFX Connect Real-Time PCR Detection System (Bio-Rad) (Di
171 Cesare et al., 2015). The specificity of the qPCR products was ensured by the analysis of the melting

profile and electrophoresis run. The standard curves for both genes were carried out by fluorometric quantification (GlowMax, Promega) and dilution of the amplicon (Di Cesare et al., 2013). Three replicates of no template control were analysed besides the samples and the standards in each qPCR assay. The limits of quantification (LQ) for 16S rDNA and *bla*$_{CTX-M}$ calculated as described in Bustin (Bustin et al., 2009), were 58 and 34 copies μL^{-1}. The mean values and standard deviations of the efficiencies and R^2 for both genes considering all the runs were 112 ± 10% and 0.99 ± 0.01, respectively. The relative abundance of the selected ARG was expressed as copy of *bla*$_{CTX-M}$ per copy of 16S rDNA gene after the conversion in copy μL^{-1} from ng μL^{-1} (Di Cesare et al., 2013). Values are reported as relative abundance of the gene or as NQ which means that the threshold cycle of the sample was below to that corresponding to LOQ but higher than the limit of the qPCR (3 copies per reaction) (40), discrepancies between replicates were treated as previously described (Di Cesare et al., 2015).

16S rRNA gene sequencing

For the DNA extracts of one year of the coastal station (June 2013 – May 2014, except August) and the daily sampling from June 2nd to 7th 2014 amplicon sequencing of the 16S rDNA gene was conducted in duplicate. Amplicons of the V3-V4 regions of the 16S rDNA gene were sequenced on an Illumina MiSeq platform (LGC Genomics) using the universal bacterial primer set S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 (Herlemann et al., 2011). The assembled paired-end raw reads were processed by a custom analysis pipeline written in DELPHI (Silva et al., 2018). It first performed a quality check using the FASTQ scores (overall average error rate per sequence <0.5), trimmed the read length to 250 bases, and performed a dereplication of identical reads. The data set was checked for chimeric sequences by a pairwise local alignment (Smith-Waterman algorithm) using the method suggested by Edgar et al (Edgar et al., 2011). The subsequent assignment of reads to OTUs was performed by an approach based on the UPARSE algorithm (Edgar, 2013). Taxonomic assignment of the OTUs was achieved by a pair-wise alignment of their representative

198 sequences to the SILVA database. Raw sequences were submitted to the Sequence Read Archive
199 (SRA) of Gene bank with BioProject ID: PRJNA505626.
200
201 Data analysis and graphs
202 All statistics were performed using the software package *R* in the R-Studio interface. Since *bla*$_{\text{CTX-M}}$
203 was often detected but not quantifiable by PCR, only presence/absence data (occurrence) was used
204 for statistical analysis. Linear models were conducted to test for a statistical relationship between the
205 water or air temperature and the occurrence of the gene.
206 Differences in cell numbers in the experimental treatments were evaluated using ANOVA with
207 Tukey's post hoc test.
208 Illumina data was rarefied to the sample with the lowest read numbers (1058 reads). β-diversity was
209 calculated using the Bray-Curtis index in the R-package "vegan" (Oksanen et al., 2007) and depicted
210 using a dendrogram with average linkage clustering. The importance of the water temperature for the
211 clustering of the microbial community was evaluated using PERMANOVA analysis using the
212 "adonis" command from the "vegan" package. A list of bacterial genera known to harbour *bla*$_{\text{CTX-M}}$
213 was retrieved from the antibiotic resistance gene database (ARDB) (Liu and Pop, 2008). Our dataset
214 was then screened for the relative abundance of these genera and their abundance patterns was
215 compared to the occurrence of *bla*$_{\text{CTX-M}}$. Figures were made in SigmaPlot 12.5 or R and composed in
216 Adobe Illustrator CS5.
217

Results

Seasonality

The occurrence of *bla*$_{CTX-M}$ was evaluated at two stations of Lake Maggiore, the pelagic station of Ghiffa (from here on referred to as pelagic station) and the coastal station of Pallanza (from here on referred to as coastal station), over the course of three consecutive years (table 1). *bla*$_{CTX-M}$ was always detectable in at least one of the stations between January and June in all years. In 2013 and 2014, between July and December the gene was never detectable at neither of the stations, whereas in 2015 it was detectable at both stations in July and at the coastal station in August, October and November. The gene was usually below the limit of quantification by qPCR, except for one sample from pelagic station and five samples from coastal station. When the gene was quantifiable, copy numbers ranged between 0.001-0.02 per 16S rDNA copy (table 1).

We tested whether the presence of the gene in the lake was related to water or air temperature (figure S1). There was a significant negative relationship between water temperature and the presence of *bla*$_{CTX-M}$ at both stations together and at the pelagic station alone (table 2, figure 1). Such a relationship was, however, not observed with the air temperature, nor was there any significant relationship between the water temperature and *bla*$_{CTX-M}$ at coastal station alone (table 2).

Short-term changes

In order to see how stable gene abundances were over a short period of time, we measured *bla*$_{CTX-M}$ daily at the coastal station, over the course of six days. The gene was always detectable but not quantifiable except for one sampling, when it suddenly reached abundances of 0.01 per mL (table 3). At the same time bacterial numbers kept constant (table 3, figure S2) and no major meteorological events occurred before the peak of *bla*$_{CTX-M}$ (figure S2).

Bacterial community

The bacterial community of the lake, as determined by amplicon sequencing of the 16S rDNA, grouped into two main clusters; one containing the samples from the autumn and winter months (from September 2013 to February 2014), where one of the two sub-clusters was formed (by December, January and February, Bray-Curtis dissimilarity, figure 2A). The second large cluster contained the Spring and Summer months including June and July 2013 and March to June 2014, where July 2013 was the most distant one to the other samples. In fact, the average water temperature explained 24% of the variance of the bray-curtis dissimilarity (PERMANOVA: $F=3.13$, $R^2=0.24$, $p=0.005$). Thus, the bacterial communities in which *bla*$_{\text{CTX-M}}$ was detected were split in two clusters one containing February and January and one containing all the other months (figure 2A).

The known potential *bla*$_{\text{CTX-M}}$ containing genera found in our dataset were *Acinetobacter*, *Escherichia/Shigella*, *Proteus*, and *Pseudomonas*. They generally had rather low read numbers within the bacterial community of the lake, with a total of 10, 54, 23 and 3, respectively. The relative abundance of most of these genera peaked in July and December 2013, two months in which *bla*$_{\text{CTX-M}}$ was not found (figure 2B). Generally, no pattern of overlap between any of the potential *bla*$_{\text{CTX-M}}$ containing genera and the gene were found in the samples from the coastal station (figure 2B).

Enrichment experiment

We then conducted an experiment to test whether the addition of cefotaxime could stimulate the growth of *bla*$_{\text{CTX-M}}$ carrying bacteria in the bacterial community of Lake Maggiore. After the first day of incubation, bacterial numbers in all treatments rose from 2×10^5 to 1.9×10^6 cells mL^{-1}, and the numbers were not significantly different between treatments (figure 3). In contrast after four days of incubation bacterial numbers were highest in the control (5.2×10^6 cells mL^{-1}) followed by the incubation with ampicillin (3.8×10^6 cells mL^{-1}) and by the incubations with cefotaxime (2.9×10^6 cells mL^{-1}, figure 3). *bla*$_{\text{CTX-M}}$ was detectable but not quantifiable at the beginning of the enrichment (table 4). After four days of incubations, the gene was still around the limit of detection or negative in all treatments (table 4) and no strains positive for *bla*$_{\text{CTX-M}}$ have been found.

DISCUSSION

The gene *bla*$_{\text{CTX-M}}$ was mainly present from January to July in the pelagic microbial community of Lake Maggiore. In fact, the gene occurrence was related to lower water temperatures, but not air temperature, suggesting a selective force within the lake. *bla*$_{\text{CTX-M}}$ was possibly harbored by specific bacterial taxa that are more abundant during winter and spring and relatively less abundant in summer and autumn. The similarity pattern of the bacterial community suggests a shift between February and March, which is likely explained by the onset of a phytoplankton spring bloom that causes a substantial turnover of the microbial community composition (Eckert et al., 2012). It is thus rather unlikely that the same microbial population harbored the *bla*$_{\text{CTX-M}}$ gene in samples from January to July. Instead, there rather appear to be at least two distinct populations, one more prominent in January and February and the other one in the spring months.

On the other hand, the presence of the gene was not directly related to water temperature at the coastal site, where it was sometimes also found in autumn and in early winter. Thus, it seems that if the water body is more directly influenced by human activities, i.e. close to the shore, the relative abundance of *bla*$_{\text{CTX-M}}$ might additionally be influenced by external factors. This hypothesis is additionally supported by the observation that the abundances of the gene suddenly spiked on a single day during the one-week sampling campaign. Since the weather, bacterial cell numbers and community composition were rather stable during that period, such a sudden increase was likely related to a point source of pollution rather than to overall changes in the microbial community. However, the currently known genera harboring *bla*$_{\text{CTX-M}}$ (Liu and Pop, 2008) did not increase in read numbers at the time points of higher gene abundances, thus a direct contamination with resistant bacteria could not be verified.

Considering that the contamination with antibiotic resistant bacteria does not seem to be the main cause of increased abundances of *bla*$_{\text{CTX-M}}$, gene abundances might increase due to contaminations with the antibiotic itself, similarly to other antibiotic and genes. For example, low concentrations of quinolones and oxytetracycline stimulated the abundances of *qnr*S and tetracycline

295 resistance genes, respectively (Knapp et al., 2008; Marti et al., 2016). To test for a stimulating effect
296 of the antibiotic on *bla*$_{\text{CTX-M}}$ we sampled water from coastal station, where the variability of the
297 abundance of this gene was higher. In our experiment, the antibiotic affected the growth of the
298 microbial community only after a few days. In fact, cell numbers increased by 10 fold during the first
299 day, independently of the presence of antibiotic. Surprisingly, little studies have centered on the effect
300 on antibiotics on the growth of microbial communities (Ding and He, 2010). One possible explanation
301 for our observation might be that the antibiotic lead to the lysis of sensitive cells, which in turn
302 released organic carbon that could be utilized by resistant cells (Leisner et al., 2016). This additional
303 substrate might have boosted initial growth, thereby compensating for the loss of bacteria due to the
304 bactericidal effect of CFX and AMP. Such a mechanism is, however, speculative and remains to be
305 tested. On the other hand, CFX did not directly select for *bla*$_{\text{CTX-M}}$ positive bacteria in the microbial
306 community of the incubations, as further proved by the absence of strains positive the gene, thus the
307 resistance was conferred by a different gene or mechanism. It is thus unlikely that the *bla*$_{\text{CTX-M}}$
308 abundances increased due to contamination with the antibiotic itself, which, in any case, likely would
309 have been at lower concentrations than those used in our incubations.
310 Altogether, these data strongly imply that *bla*$_{\text{CTX-M}}$ was naturally present in the resident
311 microbial community of Lake Maggiore for extended periods of the cold season. Increases of gene
312 abundances are likely caused by environmental parameters other than antibiotic contamination. We
313 also found no evidence for a prolonged survival of allochthonous *bla*$_{\text{CTX-M}}$ containing bacteria
314 entering the lake through wastewater. Thus, this gene may not be a good proxy for anthropogenic
315 pollution in environments similar to Lake Maggiore, as has been proposed previously (Tacão et al.,
316 2012). The phylogenetic identification of the *bla*$_{\text{CTX-M}}$ containing bacteria and evaluation of its
317 potential mobilization should be of highest priority in order to assess the risk the aquatic environment
318 might pose as a reservoir of this gene.
319

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459

460 **Table legends**

461 *Table 1.* Occurrence of the *bla*$_{\text{CTXM}}$ gene as evaluated by qPCR over the course of three years in the
462 bacterial community of Lake Maggiore. G refers to the pelagic station Ghiffa and P to the coastal
463 station Pallanza. The symbol • means that the gene was detected but below the limits for
464 quantification (NQ).
465

466 *Table 2.* Results of the linear model relating the occurrence of the *bla*$_{\text{ctxm}}$ gene to A) and C) water
467 temperature or B) and D) air temperature in both stations together and in the pelagic (Ghiffa) and
468 coastal (Pallanza) station separately.
469

Table 3. bla_{CTXM} occurrence and abundance and total bacterial abundances between Jun 2. – 7. 2014. The symbol • means that the gene was detected but below the limits for quantification (NQ).

Table 4. Occurence of the bla_{CTXM} gene as evaluated by qPCR in experimental treatments of enrichment cultures of Lake Maggiore microbial community (control), with the addition of Ampicillin or the addition of Cefotaxime.

Figure Legends

*Figure 1.*Boxplots of the water temperature of samples where bla_{CTXM} was negative(neg) or positive (pos) at the pelagic station (Ghiffa).

Figure 2. **A** Average linkage clustering of the distances of the microbial community in terms of Bray-Curtis index of the coastal sampling station in Lake Maggiore from June 2013 to June 2014. **B.** Relative abundance of reads affiliated with genera that are known as potential carriers of bla_{CTXM} in the same samples. "Total" refers to the sum of all reads of bla_{CTXM} harbouring genera.
Dates in bold print indicate that bla_{CTXM} was found then, the symbol • indicates that it was below the limits of quantification and the letter Q means that it was quantifiable (tables1&3).

Figure 3. Cell number in the incubations of enrichment cultures of Lake Maggiore microbial community (control), with the addition of Ampicillin (AMP) or the addition of Cefotaxim (CFX). Cell numbers at day 0 were $1.9x10^5$, error bars represent standard deviations of cell numbers of triplicated incubations.

Tables

Table 1. Occurrence of the *bla*$_{CTXM}$ gene as evaluated by qPCR over the course of three years in the bacterial community of Lake Maggiore. The symbol • means that the gene was detected but below the limits for quantification (NQ).

	Jan		Feb		Mar		Apr		May		Jun		Jul		Aug		Sep		Oct		Nov		Dec	
Place	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P
2013	•	•	•	•	•	•	•			•		•												
2014	Q_1	•	•	•	•	Q_2	•	•	•	•	•	•												
2015		•	•		•	•	•	Q_3	•	Q_4		•	•	Q_5		Q_6				•		•		
bla$_{CTXM}$ / 16S	Q_1= 0.005				Q_2= 0.001		Q_3= 0.004		Q_4= 0.003				Q_5= 0.03		Q_6= 0.004									

Table 2. Results of the linear model relating the occurrence of the *bla*$_{CTXM}$ gene to A) and C) water temperature or B) and D) air temperature in Ghiffa or Pallanza, respectively.

	Estimate ± ste	**t-value**	**p-value**
Ghiffa			
A) Water temperature	-0.068±0.017	-4.0	0.0003***
B) Air temperature	-0.018±0.01	-1.64	0.11
Pallanza			
C) Water temperature	-0.0387±0.02	-1.95	0.059
D) Air temperature	-0.008±0.01	-0.7	0.484

Table 3. bla$_{CTXM}$ occurrence and abundance and total bacterial abundances between Jun 2. – 7. 2014. The symbol • means that the gene was detected but below the limits for quantification (NQ).

day	***bla*$_{CTXM}$/ 16S**	**bacteria mL^{-1}**
Jun 2	•	1842000±219269
Jun 3	•	1760667±92974
Jun 4	1.13E-02	1351333±91522
Jun 5	•	1615333±79387

Jun 6	•	1130667±202372
Jun 7	•	1342000±94694

506

507 *Table 4.* Occurence of the *bla*$_{CTXM}$ gene as evaluated by qPCR in experimental treatments of
508 enrichment cultures of Lake Maggiore microbial community (control), with the addition of
509 Ampicillin or the addition of Cefotaxime.

Treatment	**T0 lake water**			**Control**			**+ Ampicillin**			**+ Cefotaxime**		
Replicate	1	2	3	1	2	3	1	2	3	1	2	3
Detectable	•	•	•	•		•	•			•		•
Negative					•			•	•		•	

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Figure

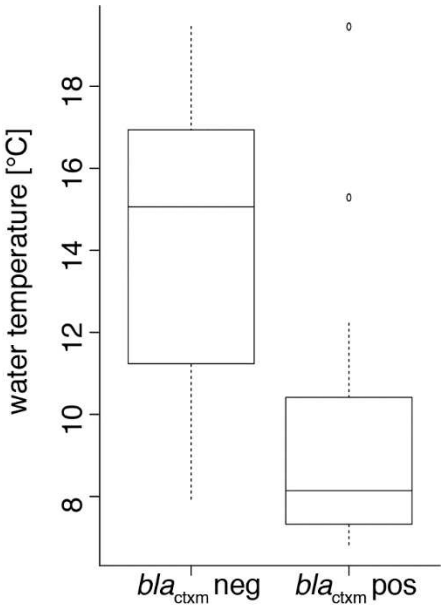
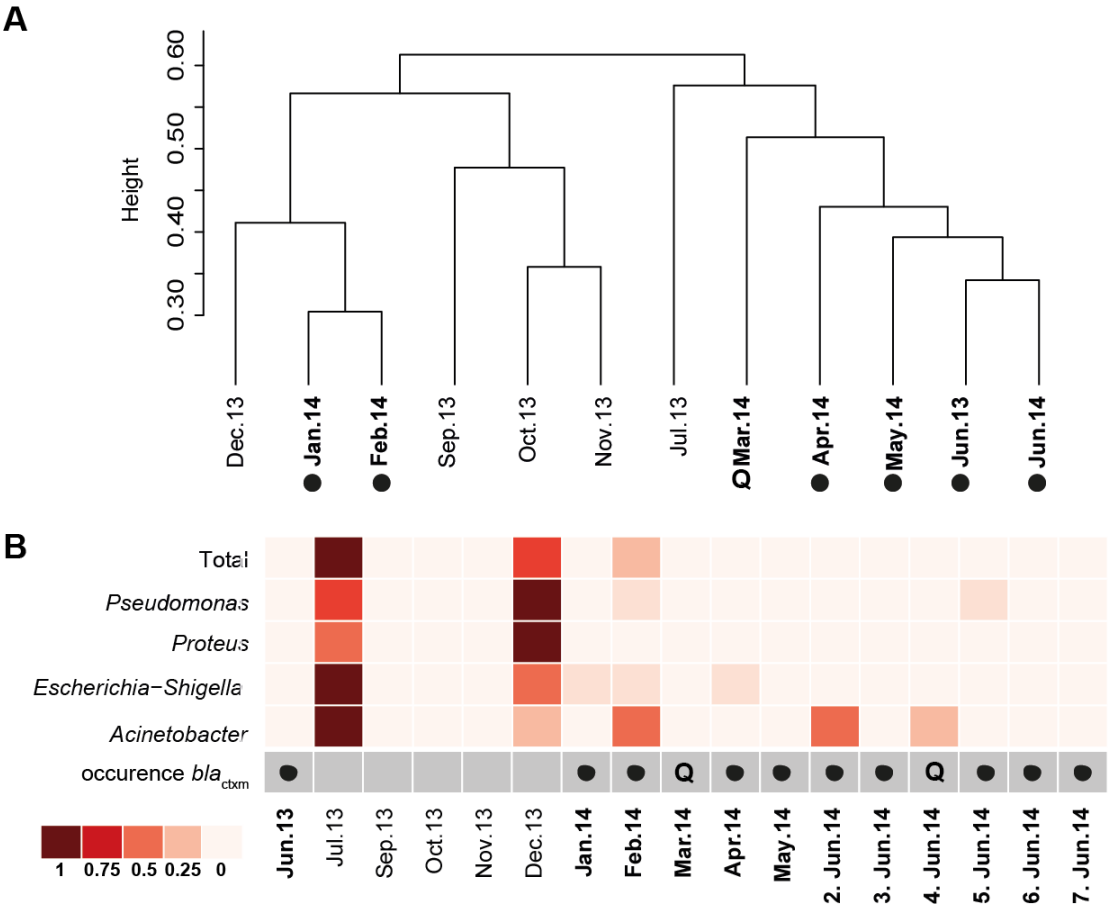


*Figure 1.*Boxplots of the water temperature of samples where *bla*$_{CTXM}$ was negative(neg) or positive (pos) at the pelagic station (Ghiffa).



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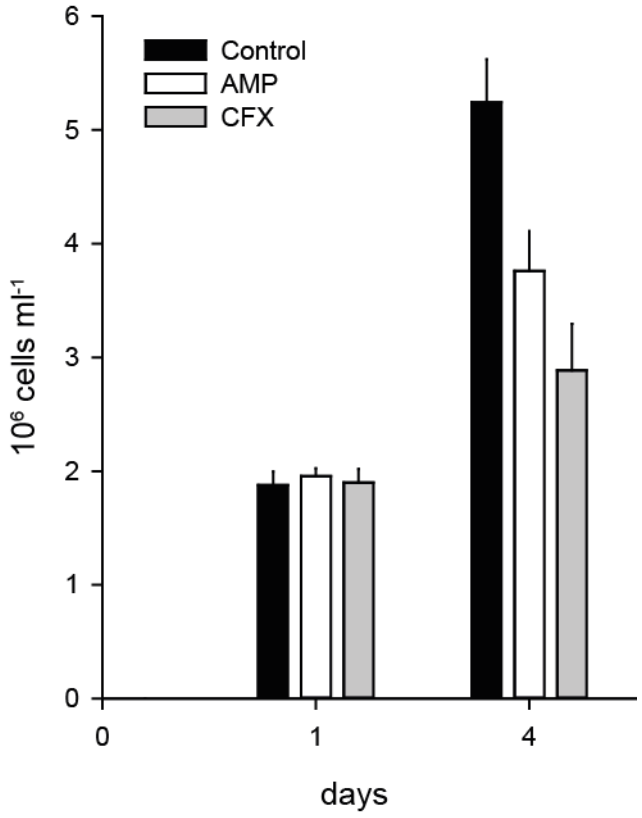

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532 triplicated incubations.

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534